Assessment of the health and antioxidant trade-off in gilthead sea bream (*Sparus aurata* L.) fed alternative diets with low levels of contaminants

Alfonso Saera-Vila[a], Laura Benedito-Palos[a], Ariadna Sitjà-Bobadilla[b], Jaime Nácher-Mestre[c], Roque Serrano[c], Sadasivam Kaushik[d], Jaume Pérez-Sánchez[a]

[a]Nutrition and Fish Growth Endocrinology Group, Institute of Aquaculture Torre de la Sal (CSIC), 12595 Ribera de Cabanes, Castellón, Spain

[b]Fish Pathology Group, Institute of Aquaculture Torre de la Sal (IATS, CSIC), 12595 Ribera de Cabanes, Castellón, Spain

[c]Research Institute for Pesticides and Water (IUPA). Avda. Sos Baynat, s/n. University Jaume I, 12071 Castellón, Spain

[d]UMR Nutrition, Aquaculture & Genomics, INRA, Unité-Mixte INRA-IFREMER-Univ. Bordeaux I, 64310 Saint-Pée-sur-Nivelle, France

Corresponding author: Jaume Pérez-Sánchez

Institute of Aquaculture Torre la Sal, 12595 Ribera de Cabanes, Castellón, Spain.

Phone: 34 964319500 Fax: 34 964319509

E-mail address: jperez@iats.csic.es

ABSTRACT

The aim of the present work was to analyze the effect of partial and total replacement of fish oil (FO) by a blend of vegetable oils on the health and antioxidant status of gilthead sea bream (*Sparus aurata* L.) fed primarily plant-protein based diets. The study included measurements of feed-borne contaminants, gene expression analyses of detoxifying and antioxidant pathways and measures of antioxidant and innate immune descriptors. Polybrominated diphenyl ethers (PBDEs) were almost undetectable in all diets, and the loading-charges of polychlorinated biphenyls (PCBs), dioxin-like PCBs, organochlorine pesticides (OCs), and polycyclic aromatic hydrocarbons (PAHs) were at trace levels decreasing their concentrations according to the level of FO replacement with vegetable oils (0%, 33%, 66%, 100%). Hepatic detoxifying pathways were down regulated by FO replacement, and the hepatic transcription of cytochrome P450 1A1 and aryl hydrocarbon receptor 1 was significantly reduced in fish fed the 100% vegetal oil diet. Dietary intervention did not alter the hepatic expression of the recycling glutathione reductase, whereas glutathione peroxidase-1 and phospholipid glutathione peroxidase were either down or up-regulated by the total FO replacement. This suggests that vegetable oils prime the *in situ* repair of peroxidized phospholipids rather than the increased turnover of membrane phospholipids from the undamaged pool of cytosolic free fatty acids. The hepatic expression of non-enzymatic antioxidants (metallothionein, glucose regulated protein 75) was down regulated in fish fed 66% and 100% vegetable oil diets. Hepatic glutathione levels and total plasma antioxidant capacity were also lowest in fish fed high levels of vegetable oils, but the concurrent increase in the GSH/GSSG ratio was interpreted as an index of reduced oxidative stress. This redox balance agrees with the enhanced respiratory burst of blood leucocytes after PMA stimulation in fish feed the 100% vegetable oil. Total plasma peroxidases and plasma alternative complement pathway were not affected by dietary treatment, whereas plasma lysozyme was significantly decreased in fish fed the 66% vegetable oil diet. Taken together, the results suggest that the health and the antioxidant status of gilthead sea bream was not damaged by high levels of FO replacement in eco-friendly diets, but both the scavenging and production of reactive oxygen species were modulated in concert by complex and nutritionally-mediated readjustments.

Key words: fish oil, vegetal oil, plant proteins, aryl hydrocarbon receptors, cytochrome P450 1A1, glucose regulated protein 75, metallothionein, glutathione, alternative complement pathway, lysozyme, peroxidases, ROS production.

1. **Introduction**

The increasing demand of fish oil (FO) to meet the expanding aquaculture industry, together with the opposing trend of fisheries and the increasing use of FO in nutraceutical and agricultural industries, has lead to the search for alternative sources of dietary lipids in fish feeds (Miller et al., 2008). Different vegetable oils at different levels of inclusion have been tested with variable results in freshwater and marine fish (Bell and Waagbo, 2008; Webster et al., 2007). Indeed, vegetable oils are rich in C_{18} polyunsaturated fatty acids (PUFA), but they are lacking in n-3 long-chain polyunsaturated fatty acids (LC-PUFAs). This means that fish feeding on vegetable oils would have to desaturate and elongate C_{18} PUFAs to their LC-PUFA derivates. However, all marine fish so far studied, including gilthead sea bream, appear to have lost the ability to make such conversion (Mourente and Tocher, 1994; Seiliez et al., 2003; Zheng et al., 2004), and therefore they have absolute dietary requirement for C_{20} and C_{22} PUFAs. On the other hand, marine derived products are also the main source of environmental pollutants, even in human dietary supplements (Storelli et al., 2004). Furthermore, the high levels found in some farmed fish have led to reconsider the possible beneficial properties of fish consumption in some population groups (Foran et al., 2005; Hamilton et al., 2005). Thus, efforts to reduce this contaminant load have also been directed towards the use of alternative vegetable oils in fish feeds (Bethune et al., 2006) and even to engineering oil seeds to produce n-3 LC-PUFA (Damude and Kinney, 2008).

The anti-inflammatory effect of n-3 LC-PUFAs has been extensively documented, and supplementation of domestic foods with marine FO is becoming an accepted practice to improve the nutritional quality of most animal products (e.g., meat, milk, eggs). However, LC-PUFAs are extremely vulnerable to oxidation and dietary antioxidants (e.g., vitamin C, vitamin E, polyphenols, carotenoids, biologically active peptides) help to counteract the

84 negative effects of lipid peroxidation, having beneficial effects on growth, fertility,
85 immunocompetence, ageing and pollutant susceptibility (Catoni et al., 2008; Erdmann et al.,
86 2008; Fang et al., 2002). In fish, most studies dealing with antioxidant systems have focused
87 on vitamin E, carotenoids and some minerals (Martínez-Alvarez et al., 2005; Mourente et al.,
88 2007a), but now there is also evidence for the antioxidant properties of plant protein
89 ingredients in practical diets for gilthead sea bream (Sitjà-Bobadilla et al., 2005), a highly
90 valuated fish for the Mediterranean aquaculture. Also, we have earlier shown that both fish
91 meal and FO can be replaced up to 65-75% without growth retardation and signs of histo-
92 pathological damage (Benedito-Palos et al., 2007; 2008). The goal of the present study is to
93 gain more understanding about the risk and benefits of these eco-friendly diets, in terms of
94 the health and anti-oxidant status of the fish. For this issue, plant protein-based diets with a
95 partial or total replacement of FO with vegetable oils were formulated, and the loading-
96 charges of the most common persistent organic pollutants (POPs) were firstly monitored. The
97 transcriptional and nutritionally-mediated effects on detoxifying and antioxidant defence
98 systems were assessed by hepatic mRNA measurements of aryl hydrocarbon receptors
99 (AhR1 and AhR2), cytochrome P450 1A1 (CYP1A; EC 1.14.14.1), metallothionein (MT),
100 glucose regulated protein 75 (GRP75), glutathione reductase (GR; EC 1.8.1.7), glutathione
101 peroxidase (GPx-1; EC 1.11.1.9) and phospholipid glutathione peroxidase (PHGPx; EC
102 1.11.1.12). Hepatic glutathione levels and total plasma antioxidant capacity were monitored
103 as antioxidant indexes. Lastly, immunological and pro-inflammatory status was assessed
104 through the alternative complement pathway, leucocyte production of reactive oxygen
105 species (ROS), and plasma measures of lysozyme and total peroxidase activities.
106

2. Materials and methods

2.1 Experimental setup

Animals and samples were the same as those described in a previous study (Benedito-Palos et al., 2008). Briefly, juvenile gilthead sea bream (*Sparus aurata* L.) of 16 g initial mean body weight were distributed into 12 fibreglass tanks (500 l) in groups of 60 fish per tank at the research experimental facilities of IATS (Castellón, Spain). Each triplicate group received from May 23rd to September 19th one of the four experimental diets nominally CTRL, 33VO, 66VO and VO (Table 1). Added oil was either Scandinavian FO (CTRL diet) or a blend of vegetable oils, replacing the 33% (33VO diet), 66% (66VO diet) and 100% (VO diet) of FO. All diets were manufactured using a twin-screw extruder at the INRA experimental research station of Donzaq (Landes, France), dried under hot air, sealed and kept in air-tight bags until use.

Fish were reared under natural day-length and water temperature following the natural changes at IATS latitude (40º 5'N; 0º 10'E). Water flow was 20 l/min and feed was offered by hand to apparent visual satiety twice a day (9.00 h-14.00 h). Each 3-4 weeks, fish were counted and group-weighed under moderate anaesthesia (3-aminobenzoic acid ethyl ester, MS 222; 100 μg/ml). There was no reduction in growth or feed efficiency (wet weight gain/dry feed intake = 1.06-1.02) with the partial replacement of FO (183-186 g final mean body weight for fish fed CTRL 33VO, 66VO diets). A decrease in feed intake and weight gain of about 10% was found with the total FO replacement (VO diet).

At the end of the feeding trial, randomly selected fish (4 fish per tank; 12 fish per treatment) were killed by a blow on the head prior to blood and tissue sampling. Blood was taken with heparinised syringes from caudal vessels, and kept on ice. One aliquot was

132 immediately used to measure respiratory burst activity of circulating leucocytes. The
133 remaining blood was centrifuged at 3000 *g* for 20 min at 4 °C, and plasma aliquots were
134 stored at -80 °C until use. Liver was extracted and rapidly excised, frozen in liquid nitrogen,
135 and stored at -80 ºC until analyses.
136
137 *2.2 Contaminant analyses*
138
139 Organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), dioxin-like
140 PCBs (DL-PCBs) and polybrominated diphenyl ethers (PBDEs) were analyzed in fish feeds
141 as described elsewhere (Serrano et al., 2003a). Briefly, feed-borne contaminants were
142 extracted by refluxing ca. 8 g during 4 h. Clean-up was performed by means of sulphuric acid
143 digestion prior to normal phase liquid chromatography (NPLC). Identification and
144 quantification of PCBs, DL-PCBs and selected OCPs were performed using a gas
145 chromatograph (GC, Varian CP-3800) coupled to a Varian Saturn 4000 ion trap mass
146 spectrometry detector (system operated in MS/MS mode). Instrumental determination of
147 PBDEs was carried out by means of a GC system (Agilent 6890N, Palo Alto, USA),
148 equipped with an autosampler (Agilent 7683) coupled to a triple quadrupole (QqQ) mass
149 spectrometer (Quattro Micro GC; Micromass, Boston, USA) operating in CI mode.
150 For polycyclic aromatic hydrocarbons (PAHs) analysis, a first saponification step was
151 carried out. PAH analytes were then extracted twice with 8 ml of n-hexane and concentrated
152 under gentle nitrogen stream at 40 °C to 1 ml. The resultant extract was purified in Florisil
153 SPE cartridge. The final extract free of interference compounds was analyzed by means of
154 the Quattro Micro GC system working in EI (MS/MS). The analytical method offered
155 satisfactory results in linearity (0.5-90 µg/ml), accuracy (recoveries between 70-120 %, n=6,

156 at 1, 10 and 20 ng/g levels), precision (RSD < 30%) and selectivity (using two transitions
157 from tandem mass spectrometry).
158 In all assays, isotopically labelled standards were added before extraction as
159 surrogates for quality control. Quantification was performed using the internal standard
160 method with external calibration curves.
161
162 *2.3 RNA extraction and RT procedure*
163
164 Total RNA extraction was performed with the ABI PRISM™ 6100 Nucleic Acid
165 PrepStation (Applied Biosystems, Foster City, CA, USA). Briefly, liver tissue was
166 homogenized at a ratio of 25 mg/ml with a guanidine-detergent lysis reagent. The reaction
167 mixture was treated with protease K, and RNA purification was achieved by passing the
168 tissue lysate (0.5 ml) through a purification tray containing an application-specific
169 membrane. Wash solutions containing DNase were applied, and total RNA was eluted into a
170 96-well PCR plate. The RNA yield was 40-50 µg with absorbance measures ($A_{260/280}$) of 1.9-
171 2.1.
172 Reverse transcription (RT) with random decamers was performed with the High-
173 Capacity cDNA Archive Kit (Applied Biosystems). For this purpose, 500 ng total RNA were
174 reverse transcribed in a final volume of 100 µl. RT reactions were incubated 10 min at 25 ºC
175 and 2 h at 37 ºC. Control reactions were run without reverse transcriptase and were used as
176 negative real-time PCR controls.
177
178

179 *2.4 Gene expression profile*

180

181 The abundance of hepatic transcript levels was analyzed by way of real-time PCR
182 assays, using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA) as
183 previously described (Calduch-Giner et al., 2003). Briefly, diluted RT reactions were
184 conveniently used for PCR reactions in 25-µl volume. Each PCR-well contained a SYBR
185 Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.3-0.9 µM to
186 obtain amplicons of 51-150 bp in length (Table 2). β-actin was used as housekeeping gene,
187 and the efficiency of PCR reactions for target and the reference gene varied between 95%
188 and 98%, respectively. The dynamic range of standard curves (serial dilutions of RT-PCR
189 reactions) spanned five orders of magnitude, and the amount of product in a particular
190 sample was determined by interpolation of the cycle threshold (Ct) value. The specificity of
191 reaction was verified by analysis of melting curves and by electrophoresis and sequencing of
192 PCR amplified products. Reactions were performed in triplicate and the fluorescence data
193 acquired during the extension phase were normalized to β-actin by the delta-delta method
194 (Livak and Schmittgen, 2001). No changes in β-actin expression were found in response to
195 dietary intervention.

196

197 *2.5 Glutathione determinations*

198

199 Frozen liver samples were homogenised with 5 volumes of ice-cold buffer [200 mM
200 2-(N-morpholino) ethanesulphonic acid, 50 mM phosphate, 1 mM EDTA, pH 6], and
201 centrifuged for 15 min at 20 000 g and 4°C. Supernatants were deproteinized with
202 metaphosphoric acid, and oxidized (GSSG) and total (tGSx) levels of glutathione were
203 determined enzimatically with a commercial kit (Cayman Chemical, Ann Arbor, MI, USA)

based on the recycling reaction of reduced glutathione (GSH) with DNTB (5,5'-dithios-2-nitrobenzoic acid) in the presence of an excess of GR. Measurements were made in a microplate reader, and the GSH/GSSG ratio was calculated as the quotient of reduced GSH equivalents.

2.6 Antioxidant capacity

Total antioxidant capacity in plasma samples was measured with a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) adapted to 96-well microplates. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS [2,2'-azino-di-(3-ethylbenzthiazoline sulphonate)] to ABTS radical cation by metamyoglobin, a derivatized form of myoglobin. The capacity of the sample to prevent ABTS oxidation is compared with that of Trolox (water-soluble tocopherol analogue), and is quantified as millimolar Trolox equivalents.

2.7 Leucocyte ROS production

Induction of the respiratory burst (RB) activity in blood leucocytes was measured directly from heparinised blood, following the method described by Nikoskelainen et al. (2005). Briefly, 100 µl of diluted blood (1:25) in HBSS (Hanks balanced salt solution, pH 7.4) were dispensed in white flat-bottomed 96-wells, and incubated with 100 µl of a freshly prepared luminol suspension (2mM luminol in 0.2 M borate buffer pH 9.0, with 2 µg/ml phorbol myristate acetate (PMA)) for 1 h at 24-25 ºC. Luminol-amplified chemiluminescence was measured every 3 min with a plate luminescence reader for generation of kinetic curves.

228 Each sample was run by duplicate and read against a blank in which no blood was added.
229 The integral luminescence in relative light units (RLU) was calculated.
230 Total plasma peroxidases (PO), which include myeloperoxidase, were chosen as a
231 measure of the oxidizing capacity of the plasma, because of their involvement in the
232 production of ROS (Spickett et al., 2000). They were measured following the procedure
233 described in Sitjà-Bobadilla et al. (2005). Briefly, 15 µl of plasma were mixed in flat-
234 bottomed well plates with 135 µl of HBSS-plus (HBSS, without Ca^{2+} and Mg^{2+}, 0.1% NaCl
235 and antimycotic/antibiotic mixture) and 50 µl of 3,3',5,5'-tetramethylbenzidine
236 hydrochloride (TMB). After 2 min of incubation, the reaction was stopped with 25 µl of 1N
237 H_2SO_4, and the optical density was read at 450 nm. Wells in which no plasma was added
238 were run as blanks.
239
240 *2.8 Lysozyme and alternative complement pathway*
241
242 The lysis by the alternative complement pathway (ACP) was determined as in Sitjà-
243 Bobadilla et al. (2005), with some modifications. Briefly, sheep red blood cells (SRBC)
244 obtained from sheep defibrinated blood (Durviz, Valencia, Spain) were used as targets at a
245 final concentration of $2.85x10^8$ cells/ml. Triplicates of tested plasma (100µl), diluted in
246 HBSS-EGTA (Hank's Balanced Salt Solution, plus 10 mM Mg^{2+} and 10 mM ethylene
247 glycolbistetra-acetate, pH 7.6), were mixed with 25 µl of SRBC in 96-well plates.
248 Microplates were then incubated for 100 min at 20ºC with constant shaking and centrifuged
249 to spin down the remaining SRBC. The absorbance of the supernatant was read at 415 nm.
250 The dilution corresponding to 50% haemolysis was expressed as ACH_{50}.
251 Plasma lysozyme was measured by a turbidimetric assay adapted to 96-well
252 microplates, as previously described (Sitjà-Bobadilla et al., 2005). Briefly, lyophilized

Micrococcus lysodeikticus (0.3 mg/ml) (Sigma) in 50 mM sodium phosphate buffer at pH 6.2 was used as a substrate for the plasma lysozyme. Triplicates of test plasma (diluted 1:2, 10 µl) were added to 200 µl of the bacterial suspension, and the reduction in absorbance at 450 nm was measured after 0.5 and 4.5 min. A unit of lysozyme activity was defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per min.

2.9 Statistical analysis

One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was used to compare means of the four different groups. The significance level was set at $P < 0.05$. All statistical analyses were performed using Sigma Stat software (SPSS Inc., IL).

3. Results

3.1 Feed-borne contaminants

Although the charge of POPs in all experimental feeds was in the range of trace levels, FO replacement with vegetable oils resulted in a significant and progressive reduction of feed-borne contaminants (Table 3). The total PCB/DL-PCB concentration on a wet weight basis ranged between 9.6 and 2.2 ng/g for the two extreme diets (CTRL and VO). Detectable amounts of HCB, DDT and DDT-derivates were found in all diets, varying the sum of these selected OCPs from 9.8 in the CTRL diet to 1.6 ng/g in the VO diet. Regardless of diet, most PBDE congeners were below the limit of detection (< 0.1 ng/g). Most PAHs were also below the limit of detection (< 0.1- 0.5 ng/g), decreasing the sum of detectable PAH congeners

(phenanthrene + anthracene, pyrene, chrysene, benzo [a] anthracene, benzo [b] fluoranthene, benzo [k] fluoranthene) from 9.5 ng/g in the CTRL diet to 4.1 ng/g in the VO diet.

3.2 Hepatic transcripts

The expression pattern of AhRs and CYP 1A is shown in Fig. 1. Hepatic transcription of AhR1 was down-regulated by FO replacement, and a significant 30% reduction in AhR1 mRNA levels was found in fish fed the VO diet (Fig. 1A). The relative expression of AhR2 was not significantly altered by dietary treatment (Fig. 1B). The trend for CYP1A was similar to that of AhR1, and a two-fold reduction in CYP1A mRNA levels was found in fish fed the VO diet (Fig. 1C).

The expression pattern of antioxidant enzymes of the hepatic glutathione pathway is shown in Fig. 2. The amount of GR mRNA transcripts was not affected by dietary treatment (Fig. 2A). The expression of GPx-1 was down-regulated by FO replacement, and a slight but significant 20% reduction in GPx-1 mRNA levels was found in fish fed the VO diet when comparisons were made with fish fed the CTRL diet (Fig. 2B). The trend for PHGPx was opposite to that of GPx-1, and the expression of PHGPx mRNA was enhanced by a 30% in fish fed the VO diet (Fig. 2C).

The hepatic expression of MT was reduced by FO replacement, and the abundance of MT mRNA levels in fish fed 66VO and VO diets was two-fold lower than in fish fed CTRL and 33VO diets (Fig. 3A). The relative expression of GRP75 was significantly reduced by a 40% in fish fed the VO diet in comparison to data found in fish fed the CTRL diet (Fig. 3B).

303 *3.3 Glutathione and antioxidant defence system*

304

305 The hepatic synthesis of glutathione was inhibited by FO replacement, and tGSx
306 levels (oxidized plus reduced forms) were significantly and progressively decreased in fish
307 fed 66VO and VO diets (Fig. 4A). The oxidized form (GSSG) also decreased with FO
308 replacement (Fig. 4B), but the GSH/GSSG ratio increased in concert and the highest quotient
309 was found in fish fed the VO diet (Fig. 4C). Dietary intervention also altered the total plasma
310 antioxidant capacity, and the values registered in fish fed the VO diet were significantly
311 lower than in CTRL fish (Fig. 5).

312

313 *3.4 Immune status*

314

315 The RB of circulating leucocytes was triggered by the graded replacement of FO.
316 Thus, ROS production after PMA stimulation was lowest in fish fed the CTRL diet and two-
317 three folds higher in fish fed the VO diet (Fig. 6A). Non significant differences were found in
318 plasma PO (Fig. 6B). Likewise, dietary intervention did not modify the ACP, although the
319 maximum ACH_{50} values (no statistically significant) were found in fish fed the 66VO diet
320 (Fig. 6C). Conversely, plasma lysozyme levels were reduced in this group of fish, and the
321 measured values in fish fed the 66VO diet were significantly lower than in the CTRL group
322 (Fig. 6D).

323

324

4. Discussion

The cardioprotective and anti-inflammatory properties (Calder, 2008) of n-3 LC-PUFAs are behind the recommendation of increased fish consumption in contemporary western diet. However, n-3 LC-PUFAs have a higher susceptibility to oxidation (Jobling and Bendiksen, 2003), and FOs and seafood in general are considered the most important source of dietary pollutants in the human diet (Abalos et al., 2008a; Borga et al., 2001; Kidd et al., 2001; Serrano et al., 2003a). In the current study, the total replacement of FO by vegetable oils reduced the total charge of POPs in fish feeds by a 45-85%. Moreover, PBDEs were below the limit of detection, and the load-charge of PCBs, DL-PCBs and OCPs was lower than or in the same range as previously reported in salmon feeds (Bell et al., 2005; Berntssen et al., 2005, Easton et al., 2002; Hites et al., 2004) and other marine fish feeds (Serrano et al., 2003b; 2008a; 2008b). In consequence, the analyzed feed-borne contaminants were at low or trace levels regardless of the diet, even in the FO diet. However, the detoxifying cytochrome/AhR pathway of gilthead sea bream appears to be sensitive enough to detect the progressive reduction of contaminants when FO was progressively reduced in fish fed 66VO and VO diets.

Cytochrome P450s constitutes a major family of drug metabolizing enzymes that transform xenobiotics to non-toxic or procarcinogenic metabolites. The transcriptional or post-transcriptional induction of CYP1A and related enzymes is mediated in gilthead sea bream (Ortiz-Delgado et al., 2002; Ortiz-Delgado and Sarasquete, 2004) and other teleosts (Barron et al., 2004; Billiard et al., 2004; Jönsson et al., 2004; Panserat et al., 2008; Yuan et al., 2006) by planar halogenated compounds (PAHs, PCBs, TCDD, etc.) that bind to cytosolic AhRs, leading to the dissociation of heat-shock protein-90 from the activated ligand-receptor complex. This specific binding ultimately results in the up-regulation of the

CYP1A gene through the activation of xenobiotic-responsive elements in the promoter region of the CYP1A gene (Hankinson, 2005). Flavonoids are thought to promote optimal health, partly via their antioxidant effects in protecting cellular components against free radicals. However, recent *in vitro* studies in mammals have also shown that some food flavonoids down-regulate CYP1A at the functional level of AhR, inhibiting the toxic effects of PCBs (Ramadass et al., 2003; Van der Heiden et al., 2009). Thus, in the current work, the significant decrease of CYP1A and AhR1 found in fish fed vegetable oils could be due not only to the reduction of contaminants, but also to the possible presence of some compounds of plant origin, such as flavonoids.

Fish differ from mammals and birds in having not one, but at least two AhR genes designated as AhR1 and AhR2. Phylogenetic analyses and gene mapping indicate that AhR1 of bony and cartilaginous fish is the ortholog of the mammalian AhR (Hahn, 2002). However, there are different roles and tissue-specific profiles for each AhR between and within fish species. Thus, AhR1 paralogs are transcribed at very low levels in Atlantic salmon, and Hansson et al. (2004) suggested that if any AhR loci become dysfunctional (pseudogenes) these should be related to AhR1. By contrast, red sea bream, which belongs to the order Perciformes (Sparidae family), shows a wide and prominent tissue expression of AhR1 (Yamauchi et al., 2005). Similarly, in the present study, the expression of AhR1 was modulated in gilthead sea bream by dietary exposure to trace levels of environmental pollutants, whereas transcript levels of AhR2 remained almost unchanged. This suggests that AhR2 is more constitutively expressed than AhR1 in sparids. However, long-term dietary exposure to PCDD/Fs, and in particular to the most toxic congener 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), clearly induced in gilthead sea bream the expression of AhR2 (Abalos et al., 2008b). Unfortunately, no data are available in this last study for AhR1 and the dual response of fish AhRs needs to be addressed in a specific and

375 dose-dependent manner not only in gilthead sea bream, but also in a wide range of sentinel
376 fish species.

377 The primary enzymatic antioxidant defence system is the glutathione redox system
378 that reduces hydrogen peroxide and lipid hydroperoxides at the expense of oxidizing GSH to
379 its disulfide form (GSSG). GR returns the oxidized glutathione form to GSH using NADPH
380 as reducing equivalents. In the present study, the hepatic expression of GR was not
381 nutritionally regulated. However, selenium-dependent glutathione peroxidases were inhibited
382 (GPx-1) or induced (PHGPx) by FO replacement according to their different substrate
383 specificity and cellular distribution, as it has been observed in other fish models (Imai and
384 Nakagawa, 2003). Thus, the cytosolic GPx-1, which acts alone or in combination with
385 phospholipase A_2 on H_2O_2 and free fatty acid hydroperoxides, was down regulated in fish fed
386 the VO diet. Conversely, mitochondrial PHGPx, which acts primarily on peroxidized fatty
387 acids of membrane phospholipids, was significantly overexpressed in the same group of fish.
388 Since these two enzymes probably operate in concert, it can be postulated that fish fed
389 vegetable oils prime the *in situ* repair of peroxidized phospholipids rather than the turnover
390 of membrane phospholipids from the cytosolic pool of undamaged free fatty acids. This
391 strategy should be especially advantageous in marine fish because their low elongase and
392 desaturase rates for bioconversion of C_{18} vegetable oils into C_{22} PUFA reduce the pool of
393 LC-PUFA in fat depots (Benedito-Palos et al., 2007; 2008).

394 Dietary intervention also modified the transcriptional regulation of non-enzymatic
395 antioxidant markers, and the VO group showed the lowest hepatic expression of GRP75 and
396 MT. The GRP75, also named mortalin/HSPA9B/PBP74/mtHSP70, is a mitochondrial-type
397 stress-protein of the heat shock protein 70 (HSP 70) family that performs a broad spectrum of
398 cellular functions, making this protein and its yeast homologue (SSC1) life-essential (Craven
399 et al., 2005; Kaul et al., 2007). Likewise, MT is a ubiquitous low molecular cystein rich-

400 protein that protects lower and higher vertebrates against heavy metals and oxidant stressors
401 (Gornati et al., 2004; Scudiero et al., 2005). Previous studies in common dentex and gilthead
402 sea bream indicate that both GRP75 and MT transcripts are regulated in these two sparid fish
403 by stressful behaviour and stress confinement, protecting fish against oxidative insults
404 (Bermejo-Nogales et al., 2007; 2008). In this way, the down regulation of these two oxidative
405 markers with FO replacement, together with the lowered plasma antioxidant capacity, can be
406 interpreted as a reduced oxidative stress. This is supported by the increased hepatic
407 GSH/GSSG ratio in VO fish despite of the reduction in absolute glutathione levels. This
408 result agrees with the recent concept that low antioxidant levels in farmed animals and long-
409 live mammals and birds predict low rates of ROS generation (Pamplona, 2008; Lykkesfeldt
410 and Svendsen, 2007).

411 Dietary fatty acids are capable of modulating the immune system in mammals (De
412 Pablo and De Cienfuegos, 2000) and n-3 LC-PUFA present in FO, and more specifically
413 eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids, have well recognized anti-
414 inflammatory properties (Calder, 2007). Conversely, many environmental toxicants
415 frequently cause inflammation by damaging tissues and inducing signalling pathways that are
416 oxidative stress-sensitive (Bols et al., 2001). Thus, FO replacement by vegetable oils could
417 provoke a double-edged effect in fish. However, in the current study, the anti-inflammatory
418 action of FO clearly over passed the possible inflammatory action of FO-borne contaminants,
419 since its replacement by vegetable oils progressively increased the PMA-induced RB of
420 blood leucocytes. This could be due to the fact that toxicant-induced inflammation usually
421 occurs at high toxicant concentrations, but not at the trace dietary levels found in the current
422 study (Pimpao et al., 2008). Similarly, the *in vitro* addition of EPA and DHA provoked a
423 marked reduction of superoxide anion generation in human polymorphonuclear leucocytes
424 (Chen et al., 1994), and dietary EPA caused a dose-dependent decrease in neutrophil RB in

425 elderly men (Rees et al., 2006). The immunosuppressive effect of n-3 LC-PUFA is thought to
426 be caused by changes in eicosanoid production, but recent studies have shown that the effects
427 of FO occur by eicosanoid-independent mechanisms, including actions upon receptor,
428 intracellular signalling pathways, transcription factor activity and gene expression (Gorjão et
429 al., 2006). However, we cannot disregard the possible inflammatory effect of vegetable
430 components. For example, the addition of soy phosphatidylcholine in human diet induced a
431 significant increase in neutrophil superoxide generation (Guarini et al., 1998), and even
432 undefined "herbal mixtures" added to fish diet appear to enhance some immune parameters,
433 including the RB (Yuan et al., 2007).

434 In fish, changing the dietary n-3 LC-PUFA levels can have both beneficial and, in
435 some instances, detrimental effects on disease resistance and immune status. Thus, the intake
436 of high levels of dietary n-3 LC-PUFA suppressed some immune functions and reduced
437 survival after pathogen challenge (Erdal et al., 1991; Fracalossi and Lovell, 1994; Kiron et
438 al., 1995; Misra et al., 2006). Conversely, other studies found significantly higher mortality
439 rates in fish fed some vegetable oils compared to those fed with FO when subsequently
440 challenged with bacteria (Bransden et al., 2003; Thompson et al., 1996). In grouper
441 (*Epinephelus malabaricus*), a high dietary DHA/EPA ratio significantly enhanced phagocytic
442 and RB activities (Wu et al., 2003), whereas 100% replacement of FO with corn oil for 8
443 weeks significantly reduced the RB of circulating leucocytes (Lin and Shiau, 2007).
444 Similarly, 40% substitution of FO by a mixture of vegetable oils, or individual vegetable oils
445 in the diet of European sea bass (*Dicentrarchus labrax*), significantly reduced the RB of head
446 kidney leucocytes (Mourente et al., 2005; 2007b). In gilthead sea bream, a 204 days-trial
447 with 60 and 80% FO replacement by single or mixed vegetable oils did not affect the RB of
448 circulating neutrophils (Montero et al., 2003). The same happened when FO was 100%
449 replaced by different vegetable oils in Atlantic salmon (*Salmo salar*) (Bell et al., 1996).

These inconsistent results concerning the effect of vegetable oils on RB may, to some extent, be due to the type of cells and the method of assessment. In our case, RB was assayed on whole blood, which avoids the mechanic impact of the isolation procedures on the cells. Furthermore, luminol-enhanced chemilumiscent is thought to measure intracellular as well as extracellular ROS, whereas other methods measure only one type of ROS production.

In the current study, total peroxidases and ACH_{50} were not significantly affected by the dietary treatment. The even levels of PO could indicate that the release of these enzymes from leucocytes was not elevated when FO was replaced. This should be regarded as potentially beneficial for fish fed vegetable oils, since high plasma MPO levels in humans are considered as a specific index of leucocytic activation in inflammatory diseases and also correlate with heart diseases and atherosclerosis (Meuwese et al., 2007; Vita et al., 2004). In accordance with the present results, ACH_{50} was not affected by 60% replacement by soybean oil (SO), rapeseed oil (RO) or linseed oil (LO) or a mixture of them when fed for a short-term to gilthead sea bream. However, when fed for a longer time (204 days), SO fed fish had significantly lower values (Montero et al., 2003). When the level of replacement was increased to 100% with SO or LO for 6 months, ACH_{50} was again decreased (Montero et al., 2008), but these effects were not seen when a mixture of LO and SO was used. By contrast, partial (but not total) replacement of FO with corn oil increased ACH_{50} in grouper (Lin and Shiau, 2007), and feeding with canola oil or with LO or safflower oil had no significant effect in the ACH_{50} of largemouth bass (*Micropterus salmoides*) (Subhadra et al., 2006), and rainbow trout (*Oncorhynchus mykiss*) (Kiron et al., 2004), respectively.

The only humoral innate factor modified by the current dietary treatment was lysozyme, which was significantly decreased in fish fed the 66VO diet. Similarly, serum lysozyme of hens fed maize oil was significantly lower than in those fed with FO (Guo et al., 2004). By contrast, serum lysozyme appears to be unaffected in most studies with fish fed

diets rich in vegetable oils (Bell et al., 1996; 2006; Kiron et al., 2004; Montero et al., 2003; Mourente et al., 2005; Subhadra et al., 2006). These apparent contradictory effects on immune factors are probably due to different experimental conditions (fish meal inclusion, type of vegetable oil, PUFAs ratios, feeding time, etc.), type of immunocytes involved and species model. One of the differences of our dietary trial is that the basal diet has also a high level of substitution of fish meal by plant proteins, and most fish trials replace either fish meal or FO.

In conclusion, the present results show that the concurrent decrease of feed-borne contaminants and inclusion of vegetable oils produced in gilthead sea bream the readjustment of detoxifying pathways and ROS production and scavenging (enzymatic and non-enzymatic antioxidants) processes. The lowering of the plasma antioxidant capacity seems not to endanger fish health, since the hepatic GSH/GSSG ratio was increased, evidencing a lower risk of lipid peroxidation and oxidative damage. Anyway, the biological significance of the lowered lysozyme or other induced changes in the defence mechanism of the fish must be further explored with experimental infections for the complete development and validation of eco-friendly aquafeeds based on the concurrent FO and fish meal replacement.

5. Acknowledgments

This work was funded by EU (FOOD-CT-2006-16249; Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers, AQUAMAX) and Spanish (Ingenio-2010 Programme; Improvement of Aquaculture Production by the use of biotechnological tools, AQUAGENOMICS) projects. AS-V was recipient of a Spanish PhD fellowship from the Diputación Provincial de Castellón.

501 **References**

502

503 Abalos, M., Parera, J., Abad, E., and Rivera, J., 2008a. PCDD/Fs and DL-PCBs in
504 feeding fats obtained as co-products or by-products derived from the food chain.
505 Chemosphere 71, 1115-1126.

506 Abalos, M., Abad, E., Estévez, A., Solé, M., Buet, A., Quirós, L., Piña, B., and Rivera,
507 J., 2008b. Effects on growth and biochemical responses in juvenile gilthead
508 seabream *Sparus aurata* after long-term dietary exposure to low levels of
509 dioxins. Chemosphere 73, S303-S310.

510 Barron, M.G., Carls, M.G., Heintz, R., and Rice, S.D., 2004. Evaluation of fish early
511 life-stage toxicity models of chronic embryonic exposures to complex polycyclic
512 aromatic hydrocarbon mixtures. Toxicol. Sci. 78, 60-67.

513 Bell, J.G., Ashton, I., Secombes, C.J., Weitzel, B.R., Dick, J.R., and Sargent, J.R., 1996.
514 Dietary lipid affects phospholipid fatty acid compositions, eicosanoid production
515 and immune function in Atlantic salmon (*Salmo salar*). Prostaglandins Leukot.
516 Essent Fatty Acids 54, 173-182.

517 Bell, J.G., McGhee, F., Dick, J.R., and Tocher, D.R., 2005. Dioxin and dioxin-like
518 polychlorinated biphenyls (PCBs) in Scottish farmed salmon (*Salmo salar*):
519 effects of replacement of dietary marine fish oil with vegetable oils. Aquaculture
520 243, 305-314.

521 Bell, J.G., Strachan, F., Good, J.E., and Tocher, D.R., 2006. Effect of dietary echium oil
522 on growth, fatty acid composition and metabolism, gill prostaglandin production
523 and macrophage activity in Atlantic cod (*Gadus morhua* L.). Aquac. Res. 37,
524 606-617.

525 Bell, J.G. and Waagbo, R., 2008. Safe and Nutritious Aquaculture Procedure: Benefits
526 and Risks of Alternative Sustainable Aquafeeds. In: Holmer,M., Black,K.,
527 Duarte,C., Marba,N., and Karakassis,I. (eds.), Aquaculture in the Ecosystem,
528 Kluwer Academic Publishers Group, pp. 185-225.

529 Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J.A., Kaushik, S., and Pérez-
530 Sánchez, J., 2007. Combined replacement of fish meal and oil in practical diets
531 for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): Networking
532 of systemic and local components of GH/IGF axis. Aquaculture 267, 199-212.

533 Benedito-Palos, L., Navarro, J.C., Sitjà-Bobadilla, A., Bell, J.G., Kaushik, S., and
534 Pérez-Sánchez, J., 2008. High levels of vegetable oils in plant protein-rich diets
535 fed to gilthead sea bream (*Sparus aurata* L.): growth performance, muscle fatty
536 acid profiles and histological alterations of target tissues. Br. J. Nutr. 100, 992-
537 1003.

538 Bermejo-Nogales, A., Saera-Vila, A., Calduch-Giner, J.A., Navarro, J.C., Sitjà-
539 Bobadilla, A., and Pérez-Sánchez, J., 2007. Differential metabolic and gene
540 expression profile of juvenile common dentex (*Dentex dentex* L.) and gilthead

541 sea bream (*Sparus aurata* L.) in relation to redox homeostasis. Aquaculture 267,
542 213-224.

543 Bermejo-Nogales, A., Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J.A., Sitjà-
544 Bobadilla, A., and Pérez-Sánchez, J., 2008. Confinement exposure induces
545 glucose regulated protein 75 (GRP75/mortalin/mtHsp70/PBP74/HSPA9B) in the
546 hepatic tissue of gilthead sea bream (*Sparus aurata* L.). Comp. Biochem.
547 Physiol. B, Biochem. Mol. Biol. 149, 428-438.

548 Berntssen, M.H.G., Lundebye, A.K., and Torstensen, B.E., 2005. Reducing the levels of
549 dioxins and dioxin-like PCBs in farmed Atlantic salmon by substitution of fish
550 oil with vegetable oil in the feed. Aquaculture Nutrition 11, 219-231.

551 Bethune, C., Seierstad, S.L., Seljeflot, I., Johansen, O., Arnesen, H., Meltzer, H.M.,
552 Rosenlund, G., Froyland, L., and Lundebye, A.K., 2006. Dietary intake of
553 differently fed salmon: a preliminary study on contaminants. Eur. J. Clin. Invest.
554 36, 193-201.

555 Billiard, S.M., Bols, N.C., and Hodson, P.V., 2004. In vitro and in vivo comparisons of
556 fish-specific CYP1A induction relative potency factors for selected polycyclic
557 aromatic hydrocarbons. Ecotoxicol. Environ. Saf. 59, 292-299.

558 Bols, N.C., Brubacher, J.L., Ganassin, R.C., and Lee, L.E.J., 2001. Ecotoxicology and
559 innate immunity in fish. Dev. Comp. Immunol. 25, 853-873.

560 Borga, K., Gabrielsen, G.W., and Skaare, J.U., 2001. Biomagnification of
561 organochlorines along a Barents Sea food chain. Environ. Pollut. 113, 187-198.

562 Bransden, M.P., Carter, C.G., and Nichols, P.D., 2003. Replacement of fish oil with
563 sunflower oil in feeds for Atlantic salmon (*Salmo salar* L.): effect on growth
564 performance, tissue fatty acid composition and disease resistance. Comp.
565 Biochem. Physiol. B, Biochem. Mol. Biol. 135, 611-625.

566 Calder, P.C., 2007. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot.
567 Essent. Fatty Acids 77, 327-335.

568 Calder, P.C., 2008. Polyunsaturated fatty acids, inflammatory processes and
569 inflammatory bowel diseases. Mol Nutr Food Res 52, 885-897.

570 Calduch-Giner, J.A., Mingarro, M., Vega-Rubín de Celis, S., Boujard, D., and Pérez-
571 Sánchez, J., 2003. Molecular cloning and characterization of gilthead sea bream,
572 (*Sparus aurata*) growth hormone receptor (GHR). Assessment of alternative
573 splicing. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 136, 1-13.

574 Catoni, C., Peters, A., and Martin Schaefer, H., 2008. Life history trade-offs are
575 influenced by the diversity, availability and interactions of dietary antioxidants.
576 Anim Behav 76, 1107-1119.

577 Chen, L.Y., Lawson, D.L., and Mehta, J.L., 1994. Reduction in human neutrophil
578 superoxide anion generation by n-3 polyunsaturated fatty-acids - Role of
579 cyclooxygenase products and endothelium-derived relaxing factor. Thromb.
580 Res. 76, 317-322.

581 Craven, S.E., French, D., Ye, W., de Sauvage, F., and Rosenthal, A., 2005. Loss of
582 Hspa9b in zebrafish recapitulates the ineffective hematopoiesis of the
583 myelodysplastic syndrome. Blood 105, 3528-3534.

584 Damude, H.G. and Kinney, A.J., 2008. Engineering oilseeds to produce nutritional fatty
585 acids. Physiol Plant 132, 1-10.

586 De Pablo, M.A. and De Cienfuegos, G.A., 2000. Modulatory effects of dietary lipids on
587 immune system functions. Immunol. Cell Biol. 78, 31-39.

588 Easton, M.D.L., Luszniak, D., and Von der Geest, E., 2002. Preliminary examination of
589 contaminant loadings in farmed salmon, wild salmon and commercial salmon
590 feed. Chemosphere 46, 1053-1074.

591 Erdal, J.I., Evensen, O., Kaurstad, O.K., Lillehaug, A., Solbakken, R., and Thorud, K.,
592 1991. Relationship between diet and immune response in Atlantic salmon
593 (*Salmo salar* L) after feeding various levels of ascorbic-acid and omega-3-fatty-
594 acids. Aquaculture 98, 363-379.

595 Erdmann, K., Cheung, B.W.Y., and Schröder, H., 2008. The possible roles of food-
596 derived bioactive peptides in reducing the risk of cardiovascular disease. J. Nutr.
597 Biochem. 19, 643-654.

598 Fang, Y.Z., Yang, S., and Wu, G., 2002. Free radicals, antioxidants, and nutrition.
599 Nutrition 18, 872-879.

600 Foran, J.A., Good, D.H., Carpenter, D.O., Hamilton, M.C., Knuth, B.A., and Schwager,
601 S.J., 2005. Quantitative analysis of the benefits and risks of consuming farmed
602 and wild salmon. J. Nutr. 135, 2639-2643.

603 Fracalossi, D.M. and Lovell, R.T., 1994. Dietary lipid sources influence responses of
604 channel catfish (*Ictalurus punctatus*) to challenge with the pathogen
605 *Edwardsiella ictaluri*. Aquaculture 119, 287-298.

606 Gorjão, R., Verlengia, R., Lima, T.M.d., Soriano, F.G., Boaventura, M.F.C., Kanunfre,
607 C.C., Peres, C.M., Sampaio, S.C., Otton, R., Folador, A., Martins, E.F., Curi,
608 T.C.P., Portiolli, É.P., Newsholme, P., and Curi, R., 2006. Effect of
609 docosahexaenoic acid-rich fish oil supplementation on human leukocyte
610 function. Clin Nutr 25, 923-938.

611 Gornati, R., Papis, E., Rimoldi, S., Terova, G., Saroglia, M., and Bernardini, G., 2004.
612 Rearing density influences the expression of stress-related genes in sea bass
613 (*Dicentrarchus labrax*, L.). Gene 341, 111-118.

614 Guarini, P., Bellavite, P., Biasi, D., Carletto, A., Galvani, S., Caramaschi, P., Bambara,
615 L.M., and Corrocher, R., 1998. Effects of dietary fish oil and soy
616 phosphatidylcholine on neutrophil fatty acid composition, superoxide release,
617 and adhesion. Inflammation 22, 381-391.

618 Guo, Y.M., Chen, S.Y., Xia, Z.G., and Yuan, J.M., 2004. Effects of different types of
619 polyunsaturated fatty acids on immune function and PGE(2) synthesis by

620 peripheral blood leukocytes of laying hens. Anim. Feed Sci. Technol. 116, 249-
621 257.

622 Hahn, M.E., 2002. Aryl hydrocarbon receptors: diversity and evolution. Chem. Biol.
623 Interact.141, 131-160.

624 Hamilton, M.C., Hites, R.A., Schwager, S.J., Foran, J.A., Knuth, B.A., and Carpenter,
625 D.O., 2005. Lipid composition and contaminants in farmed and wild salmon.
626 Environ. Sci. Technol. 39, 8622-8629.

627 Hankinson, O., 2005. Role of coactivators in transcriptional activation by the aryl
628 hydrocarbon receptor. Arch. Biochem. Biophys. 433, 379-386.

629 Hansson, M.C., Wittzell, H., Persson, K., and von Schantz, T., 2004. Unprecedented
630 genomic diversity of AhR1 and AhR2 genes in Atlantic salmon (*Salmo salar*
631 L.). Aquat. Toxicol. 68, 219-232.

632 Hites, R.A., Foran, J.A., Carpenter, D.O., Hamilton, M.C., Knuth, B.A., and Schwager,
633 S.J., 2004. Global assessment of organic contaminants in farmed salmon.
634 Science 303, 226-229.

635 Imai, H. and Nakagawa, Y., 2003. Biological significance of phospholipid
636 hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. Free
637 Radic. Biol. Med. 34, 145-169.

638 Jobling, M. and Bendiksen, E.A., 2003. Dietary lipids and temperature interact to
639 influence tissue fatty acid compositions of Atlantic salmon, *Salmo salar* L., parr.
640 Aquac. Res. 34, 1423-1441.

641 Jönsson, M.E., Brunström, B., Ingebrigtsen, K., and Brandt, I., 2004. Cell-specific
642 CYP1A expression and benzo[a]pyrene adduct formation in gills of rainbow
643 trout (*Oncorhynchus mykiss*) following CYP1A induction in the laboratory and
644 in the field. Environ. Toxicol. Chem. 23, 874-882.

645 Kaul, S.C., Deocaris, C.C., and Wadhwa, R., 2007. Three faces of mortalin: A
646 housekeeper, guardian and killer. Exp. Gerontol. 42, 263-274.

647 Kidd, K.A., Bootsma, H.A., Hesslein, R.H., Muir, D.C.G., and Hecky, R.E., 2001.
648 Biomagnification of DDT through the benthic and pelagic food webs of lake
649 malawi, east Africa: importance of trophic level and carbon source. Environ.
650 Sci. Technol. 35, 14-20.

651 Kiron, V., Fukuda, H., Takeuchi, T., and Watanabe, T., 1995. Essential fatty-acid
652 nutrition and defense mechanisms in rainbow trout *Oncorhynchus mykiss*. Comp
653 Biochem Physiol A Comp Physiol 111, 361-367.

654 Kiron, V., Puangkaew, J., Ishizaka, K., Satoh, S., and Watanabe, T., 2004. Antioxidant
655 status and nonspecific immune responses in rainbow trout (*Oncorhynchus*
656 *mykiss*) fed two levels of vitamin E along with three lipid sources. Aquaculture
657 234, 361-379.

658 Lin, Y.H. and Shiau, S.Y., 2007. Effects of dietary blend of fish oil with corn oil on
659 growth and non-specific immune responses of grouper, *Epinephelus*
660 *malabaricus*. Aquaculture Nutrition 13, 137-144.

661 Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using
662 real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. Methods 25, 402-408.

663 Lykkesfeldt, J. and Svendsen, O., 2007. Oxidants and antioxidants in disease: Oxidative
664 stress in farm animals. The Veterinary Journal 173, 502-511.

665 Martínez-Alvarez, R., Morales, A., and Sanz, A., 2005. Antioxidant defenses in fish:
666 Biotic and Abiotic Factors. Rev. Fish Biol. Fish. 15, 75-88.

667 Meuwese, M.C., Stroes, E.S.G., Hazen, S.L., van Miert, J.N., Kuivenhoven, J.A.,
668 Schaub, R.G., Wareham, N.J., Luben, R., Kastelein, J.J.P., Khaw, K.T., and
669 Boekholdt, S.M., 2007. Serum Myeloperoxidase levels are associated with the
670 future risk of coronary artery disease in apparently healthy individuals - The
671 EPIC-Norfolk prospective population study. J. Am. Coll. Cardiol. 50, 159-165.

672 Miller, M.R., Nichols, P.D., and Carter, C.G., 2008. n-3 Oil sources for use in
673 aquaculture - alternatives to the unsustainable harvest of wild fish. Nutr Res Rev
674 21, 85-96.

675 Misra, S., Sahu, N.P., Pal, A.K., Xavier, B., Kumar, S., and Mukherjee, S.C., 2006. Pre-
676 and post-challenge immuno-haematological changes in *Labeo rohita* juveniles
677 fed gelatinised or non-gelatinised carbohydrate with n-3 PUFA. Fish Shellfish
678 Immunol. 21, 346-356.

679 Montero, D., Grasso, V., Izquierdo, M.S., Ganga, R., Real, F., Tort, L., Caballero, M.J.,
680 and Acosta, F., 2008. Total substitution of fish oil by vegetable oils in gilthead
681 sea bream (*Sparus aurata*) diets: Effects on hepatic Mx expression and some
682 immune parameters. Fish Shellfish Immunol. 24, 147-155.

683 Montero, D., Kalinowski, T., Obach, A., Robaina, L., Tort, L., Caballero, M.J., and
684 Izquierdo, M.S., 2003. Vegetable lipid sources for gilthead seabream (*Sparus*
685 *aurata*): effects on fish health. Aquaculture 225, 353-370.

686 Mourente, G. and Tocher, D.R., 1994. In-vivo metabolism of [1-C-14]Linolenic Acid
687 (18/3(N-3)) and [1-C-14]Eicosapentaenoic Acid (20/5(N-3)) in a marine fish -
688 Time-course of the desaturation/elongation pathway. Biochim. Biophys. Acta-
689 Lipids and Lipid Metabolism 1212, 109-118.

690 Mourente, G., Good, J.E., and Bell, J.G., 2005. Partial substitution of fish oil with
691 rapessed, linseed and olive oils in diets for European sea bass (*Dicentrarchus*
692 *labrax L.)*: effects on flesh fatty acid composition, plasma prostaglandins and E_2
693 F_{2a}, immue function and effectiveness of a fish oil finishing diet. Aquaculture
694 Nutrition 11, 25-40.

695 Mourente, G., Bell, J.G., and Tocher, D.R., 2007a. Does dietary tocopherol level affect
696 fatty acid metabolism in fish? Fish Physiol. Biochem. 33, 269-280.

697 Mourente, G., Good, J.E., Thompson, K.D., and Bell, J.G., 2007b. Effects of partial
698 substitution of dietary fish oil with blends of vegetable oils, on blood leucocyte
699 fatty acid compositions, immune function and histology in European sea bass
700 (*Dicentrarchus labrax* L.). Br. J. Nutr. 98, 770-779.

701 Nikoskelainen, S., Verho, S., Airas, K., and Lilius, E.M., 2005. Adhesion and ingestion
702 activities of fish phagocytes induced by bacterium *Aeromonas salmonicida* can
703 be distinguished and directly measured from highly diluted whole blood of fish.
704 Dev. Comp. Immunol. 29, 525-537.

705 Ortiz-Delgado, J.B., Sarasquete, C., Behrens, A., González de Canales, M.L., and
706 Segner, H., 2002. Expression, cellular distribution and induction of cytochrome
707 P4501A (CYP1A) in gilthead seabream, *Sparus aurata*, brain. Aquat. Toxicol.
708 60, 269-283.

709 Ortiz-Delgado, J.B. and Sarasquete, C., 2004. Toxicity, histopathological alterations and
710 immunohistochemical CYP1A induction in the early life stages of the seabream,
711 *Sparus aurata*, following waterbone exposute to B(a)P and TCDD. J. Mol.
712 Histol. 35, 29-45.

713 Pamplona, R., 2008. Membrane phospholipids, lipoxidative damage and molecular
714 integrity: A causal role in aging and longevity. Biochim. Biophys. Acta -
715 Bioenergetics 1777, 1249-1262.

716 Panserat, S., Kolditz, C., Richard, N., Plagnes-Juan, E., Piumi, F., Esquerré, D., Medale,
717 F., Corraze, G., and Kaushik, S., 2008. Hepatic gene expression profiles in
718 juvenile rainbow trout (*Oncorhynchus mykiss*) fed fishmeal or fish oil-free diets.
719 Br. J. Nutr. 100, 953-957.

720 Pimpao, C.T., Zampronio, A.R., and Silva de Assis, H.C., 2008. Exposure of *Ancistrus*
721 *multispinis* (Regan, 1912, Pisces, Teleostei) to deltamethrin: Effects on cellular
722 immunity. Fish Shellfish Immunol. 25, 528-532.

723 Ramadass, P., Meerarani, P., Toborek, M., Robertson, L.W., and Hennig, B., 2003.
724 Dietary flavonoids modulate PCB-induced oxidative stress, CYP1A1 induction,
725 and AhR-DNA binding activity in vascular endothelial cells. Toxicol. Sci. 76,
726 212-219.

727 Rees, D., Miles, E.A., Banerjee, T., Wells, S.J., Roynette, C.E., Wahle, K.W.J., and
728 Calder, P.C., 2006. Dose-related effects of eicosapentaenoic acid on innate
729 immune function in healthy humans: a comparison of young and older men. Am.
730 J. Clin. Nutr. 83, 331-342.

731 Scudiero, R., Temussi, P.A., and Parisi, E., 2005. Fish and mammalian
732 metallothioneins: a comparative study. Gene 345, 21-26.

733 Seiliez, I., Panserat, S., Corraze, G., Kaushik, S., and Bergot, P., 2003. Cloning and
734 nutritional regulation of a [Delta]6-desaturase-like enzyme in the marine teleost
735 gilthead seabream (*Sparus aurata*). Comp. Biochem. Physiol. B, Biochem. Mol.
736 Biol. 135, 449-460.

737 Serrano, R., Barreda, M., Pitarch, E., and Hernández, F., 2003a. Determination of low
738 concentrations of organochlorine pesticides and PCBs in fish feed and fish
739 tissues from aquaculture activities by gas chromatography with tandem mass
740 spectrometry. J Sep Sci 26, 75-86.

741 Serrano, R., Simal-Julian, A., Pitarch, E., Hernandez, F., Varo, I., and Navarro, J.C.,
742 2003b. Biomagnification study on organochlorine compounds in marine
743 aquaculture: the sea bass (*Dicentrarchus labrax*) as a model. Environ. Sci.
744 Technol. 37, 3375-3381.

745 Serrano, R., Barreda, M., and Blanes, M.A., 2008a. Investigating the presence of
746 organochlorine pesticides and polychlorinated biphenyls in wild and farmed
747 gilthead sea bream (*Sparus aurata*) from the Western Mediterranean sea. Mar.
748 Pollut. Bull. 56, 963-972.

749 Serrano, R., Blanes, M.A., and López, F.J., 2008b. Maternal transfer of organochlorine
750 compounds to oocytes in wild and farmed gilthead sea bream (*Sparus aurata*).
751 Chemosphere 70, 561-566.

752 Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., and
753 Pérez-Sánchez, J., 2005. Effect of fish meal replacement by plant protein
754 sources on non-specific defence mechanisms and oxidative stress in gilthead sea
755 bream (*Sparus aurata*). Aquaculture 249, 387-400.

756 Spickett, C.M., Jerlich, A., Panasenko, O.M., Arnhold, J., Pitt, A.R., Stelmaszynska, T.,
757 and Schaur, R.J., 2000. The reactions of hypochlorous acid, the reactive oxygen
758 species produced by myeloperoxidase, with lipids. Acta Biochim. Pol. 47, 889-
759 899.

760 Storelli, M.M., Storelli, A., and Marcotrigiano, G.O., 2004. Polychlorinated biphenyls,
761 hexachlorobenzene, hexachlorocyclohexane isomers, and pesticide
762 organochlorine residues in cod-liver oil dietary supplements. J. Food Prot. 67,
763 1787-1791.

764 Subhadra, B., Lochmann, R., Rawles, S., and Chen, R.G., 2006. Effect of dietary lipid
765 source on the growth, tissue composition and hematological parameters of
766 largemouth bass (*Micropterus salmoides*). Aquaculture 255, 210-222.

767 Thompson, K.D., Tatner, M.F., and Henderson, R.J., 1996. Effects of dietary (n-3) and
768 (n-6) polyunsaturated fatty acid ratio on the immune response of Atlantic
769 salmon, *Salmo salar* L. Aquaculture Nutrition 2, 21-31.

770 Van der Heiden, E., Bechoux, N., Muller, M., Sergent, T., Schneider, Y.J., Larondelle,
771 Y., Maghuin-Rogister, G., and Scippo, M.L., 2009. Food flavonoid aryl
772 hydrocarbon receptor-mediated agonistic/antagonistic/synergic activities in
773 human and rat reporter gene assays. Anal. Chim. Acta 637, 337-345.

774 Vita, J.A., Brennan, M.L., Gokce, N., Mann, S.A., Goormastic, M., Shishehbor, M.H.,
775 Penn, M.S., Keaney, J.F., Jr., and Hazen, S.L., 2004. Serum myeloperoxidase
776 levels independently predict endothelial dysfunction in humans. Circulation 110,
777 1134-1139.

778 Webster,C., Lim,C., and Lee,S.-C., 2007. Use of alternative protein sources in
779 aquaculture diets. The Haworth Press, Inc, NY, USA.

780 Wu, F.C., Ting, Y.Y., and Chen, H.Y., 2003. Dietary docosahexaenoic acid is more
781 optimal than eicosapentaenoic acid affecting the level of cellular defence
782 responses of the juvenile grouper *Epinephelus malabaricus*. Fish Shellfish
783 Immunol. 14, 223-238.

784 Yamauchi, M., Kim, E.Y., Iwata, H., and Tanabe, S., 2005. Molecular characterization
785 of the aryl hydrocarbon receptors (AHR1 and AHR2) from red seabream
786 (*Pagrus major*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141, 177-187.

787 Yuan, C., Li, D., Chen, W., Sun, F., Wu, G., Gong, Y., Tang, J., Shen, M., and Han, X.,
788 2007. Administration of a herbal immunoregulation mixture enhances some
789 immune parameters in carp (*Cyprinus carpio*). Fish Physiol. Biochem. 33, 93-
790 101.

791 Yuan, Z., Courtenay, S., and Wirgin, I., 2006. Comparison of hepatic and extra hepatic
792 induction of cytochrome P4501A by graded doses of aryl hydrocarbon receptor
793 agonists in Atlantic tomcod from two populations. Aquat. Toxicol. 76, 306-320.

794 Zheng, X., Seiliez, I., Hastings, N., Tocher, D.R., Panserat, S., Dickson, C.A., Bergot,
795 P., and Teale, A.J., 2004. Characterization and comparison of fatty acyl Δ6
796 desaturase cDNAs from freshwater and marine teleost fish species. Comp.
797 Biochem. Physiol. B, Biochem. Mol. Biol. 139, 269-279.
798
799

800

801 **Figure Legends**

802

803 Figure 1. Effects of dietary treatment on hepatic transcript levels of aryl hydrocarbon
804 receptor 1 (A), aryl hydrocarbon receptor 2 (B) and cytochrome P450 1A1 (C). Data in fish
805 fed the control diet were used as arbitrary reference values in the normalization procedure
806 (values > 1 or < 1 indicate increase or decrease respect to reference values). Different letters
807 indicate significant differences ($P< 0.05$) among dietary treatments (Student-Newman-Keuls
808 test).

809

810 Figure 2. Effects of dietary treatment on hepatic transcript levels of glutathione reductase
811 (A), glutathione peroxidase (B) and phospholipid glutathione peroxidase (C). Data in fish fed
812 the control diet were used as arbitrary reference values in the normalization procedure
813 (values > 1 or < 1 indicate increase or decrease respect to reference values). Different letters
814 indicate significant differences ($P< 0.05$) among dietary treatments (Student-Newman-Keuls
815 test).

816

817 Figure 3. Effects of dietary treatment on hepatic transcript levels of metallothionein (A) and
818 glucose regulated protein 75 (A). Data in fish fed the control diet were used as arbitrary
819 reference values in the normalization procedure (values > 1 or < 1 indicate increase or
820 decrease respect to reference values). Different letters indicate significant differences ($P<$
821 0.05) among dietary treatments (Student-Newman-Keuls test).

822

823 Figure 4. Effects of dietary treatment on hepatic glutathione levels. Total glutathione (A),
824 oxidized glutathione (B) and reduced oxidized glutathione ratio (C). Different letters

825 indicate significant differences ($P < 0.05$) among dietary treatments (Student-Newman-Keuls
826 test).
827
828 Figure 5. Effects of dietary treatment on the total plasma antioxidant capacity. Different
829 letters indicate significant differences ($P < 0.05$) among dietary treatments (Student-Newman-
830 Keuls test).
831
832 Figure 6. Effects of dietary treatment on the respiratory burst of circulating leucocytes (A),
833 plasma peroxidases (B), serum alternative complement pathway (C) and plasma lyzozyme
834 levels (D). Different letters indicate significant differences ($P < 0.05$) among dietary
835 treatments (Student-Newman-Keuls test).
836

837 **Table 1.** Ingredients and chemical composition of experimental diets. For details in amino
838 acid and fatty acid composition see Benedito-Palos et al. (2007).
839

Ingredient (g/kg)	CTRL	33VO	66VO	VO
Fish meal (CP 70%) [1]	150	150	150	150
CPSP 90 [2]	50	50	50	50
Corn gluten	400	400	400	400
Soybean meal	143	143	143	143
Extruded wheat	40	40	40	40
Fish oil [3]	151.5	101.5	51.5	0
Rapeseed oil	0	8.5	17	25.8
Linseed oil	0	29	58	87.9
Palm oil	0	12.5	25	37.9
Soya lecithin	10	10	10	10
Binder	10	10	10	10
Mineral premix [4]	10	10	10	10
Vitamin premix [5]	10	10	10	10
$CaHPO_4.2H_2O$ (18%P)	20	20	20	20
L-Lys	5.5	5.5	5.5	5.5
Proximate composition				
Dry matter (DM, %)	93.42	94.16	94.79	95.38
Protein (% DM)	48.98	48.74	49.03	48.65
Fat (% DM)	22.19	22.26	22.11	22.31
Ash (% DM)	6.54	6.57	6.62	6.41

840
841 [1]Fish meal (Scandinavian LT)
842 [2]Fish soluble protein concentrate (Sopropêche, France)
843 [3]Fish oil (Sopropêche, France)
844 [4]Supplied the following (mg / kg diet, except as noted): calcium carbonate (40% Ca) 2.15 g,
845 magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric citrate 0.2 g, potassium
846 iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen phosphate 50 g, copper sulphate 0.3, zinc
847 sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0.3.
848 [5]Supplied the following (mg / kg diet): retinyl acetate 2.58, DL-cholecalciferol 0.037, DL-α
849 tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5, riboflavin 15, pyridoxine 7.5,
850 nicotinic acid 87.5, folic acid 2.5, calcium pantothenate 2.5, vitamin B_{12} 0.025, ascorbic acid 250,
851 inositol 500, biotin 1.25 and choline chloride 500.
852

Table 2. Forward and reverse primers for hepatic real-time PCR assays. Aryl hydrocarbon receptors (AhR1, AhR2), cytochrome P450 1A1 (CYP1A), metallothionein (MT), glucose regulated protein 75 (GRP75), glutathione reductase (GR), glutathione peroxidase (GPx-1), phospholipid glutathione peroxidase (PHGPx) and ß-actin.

Gene	GenBank accession		Primer sequence	Position
AhR1	EU254480	F	CCT GGG ACT GAA CGC CGA AG	1027-1046
		R	GCT AAG TGT TGG GAT GTG GTT GG	1120-1098
AhR2	AY129956	F	TCA GAG GGA TTG GTG TTT TAT GTC	358-381
		R	TGG GTT TAG AGC AAA GTG AAG C	507-486
CYP1A	AFO11223	F	GCA TCA ACG ACC GCT TCA ACG C	903-924
		R	CCT ACA ACC TTC TCA TCC GAC ATC TGG	1071-1045
GR	AJ937873	F	TGT TCA GCC ACC CAC CCA TCG G	927-948
		R	GCG TGA TAC ATC GGA GTG AAT GAA GTC TTG	1041-1012
GPx-1	DQ524992	F	GAA GGT GGA TGT GAA TGG AAA AGA TG	34-59
		R	CTG ACG GGA CTC CAA ATG ATG G	162-141
PHGPx	AM977818	F	TGC GTC TGA TAG GGT CCA CTG TC	237-259
		R	GTC TGC CAG TCC TCT GTC GG	312-293
MT	U93206	F	CTC TAA GAC TGG AAC CTG	75-92
		R	GGG CAG CAT GAG CAG CAG	167-150
GRP75	DQ524993	F	TCC GGT GTG GAT CTG ACC AAA GAC	358-381
		R	TGT TTA GGC CCA GAA GCA TCC ATG	500-477
ß-actin	X89920	F	TCC TGC GGA ATC CAT GAG A	811-829
		R	GAC GTC GCA CTT CAT GAT GCT	861-841

Table 3. Concentration of PCBs, DL-PCBs (*), OCPs, PBDEs and PAHs in experimental diets. Each value is the mean of three separate determinations. Coefficient of variation is under parentheses.

Compound (ng/g wet wt)	CTRL	33VO	66VO	VO
PCB 28+31	0.2(3)	0.1(3)	0.1(3)	< 0.1
PCB 52	0.5(5)	0.3(6)	0.2(4)	0.1(9)
PCB 101	1(13)	0.7(5)	0.4(10)	0.2(10)
PCB 77*	< 0.2	< 0.2	< 0.2	< 0.2
PCB 118*	1(5)	0.8(11)	0.5(12)	0.2(21)
PCB 153	2.5(5)	2(7)	1.5(5)	0.6(4)
PCB 105*	0.4(2)	0.3(21)	0.2(8)	< 0.1
PCB 138	1.4(2)	1.3(3)	0.8(15)	0.4(5)
PCB 126*	< 0.2	< 0.2	< 0.2	< 0.2
PCB 128	0.3(30)	0.2(4)	0.2(7)	< 0.1
PCB 156 *	0.3(28)	0.2(16)	< 0.1	0.1(25)
PCB 180	0.7(6)	0.8(21)	0.5(3)	0.3(15)
PCB 169*	0.2(10)	0.1(18)	< 0.1	< 0.1
PCB 170	0.3(5)	0.3(11)	0.2(12)	< 0.2
HCB	0.6(6)	0.4(10)	0.4(10)	0.2(11)
p,p´-DDT	5.8(8)	4.1(4)	2.6(3)	0.9(12)
p,p´-DDE	2.2(7)	1.7(5)	1.1(11)	0.3(27)
p,p´-DDD	1.2(6)	1.1(12)	0.7(7)	0.3(24)
PBDE 28	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 71	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 47	0.13(11)	0. 12(12)	< 0.1	< 0.1
PBDE 66	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 100	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 99	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 85	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 154	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 153	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 138	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 183	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 209	< 0.1	< 0.1	< 0.1	< 0.1
Naphthalene	< 0.5	< 0.5	< 0.5	< 0.5
Acenaphthylene	< 0.1	< 0.2	< 0.2	< 0.2
Acenaphthene	< 0.2	< 0.2	< 0.2	< 0.2
Fluorene	< 0.3	< 0.3	< 0.3	< 0.3
Phenanthrene +Anthracene	0.62(15)	1.50(5)	0.38(11)	< 0.2
Fluoranthene	< 0.2	< 0.2	< 0.2	< 0.2
Pyrene	1.33(7)	0.83(15)	< 0.2	< 0.2
Benzo [a] anthracene	1.12(5)	< 0.2	< 0.2	< 0.2
Chrysene	1.41(5)	< 0.2	< 0.2	< 0.2
Benzo [b] fluoranthene	2.68(4)	2.01(19)	2.20(5)	2.06(8)
Benzo [k] fluoranthene	2.31(8)	2.19(7)	2.16(7)	2.07(6)
Benzo [a] pyrene	< 0.2	< 0.2	< 0.2	< 0.2
Indeno [1,2,3-cd] pyrene	< 0.2	< 0.2	< 0.2	< 0.2
Dibenzo [a,h] anthracene	< 0.2	< 0.2	< 0.2	< 0.2
Benzo [g,h,i] perylene	< 0.2	< 0.2	< 0.2	< 0.2
∑*PCBs + DL-PCBs*	8.66	7.03	4.71	2.22
∑*OCPs*	9.8	7.3	4.8	1.6
∑*PAHs*	9.5	6.5	4.7	4.1
DL-PCBs TEQ, ng/g wet wt to humans	0.00231	0.00123	0.00114	0.0011
DL-PCBs TEQ, ng/g wet wt to fish	0.0000385	0.0000315	0.000029	0.000027

888

Figure 1

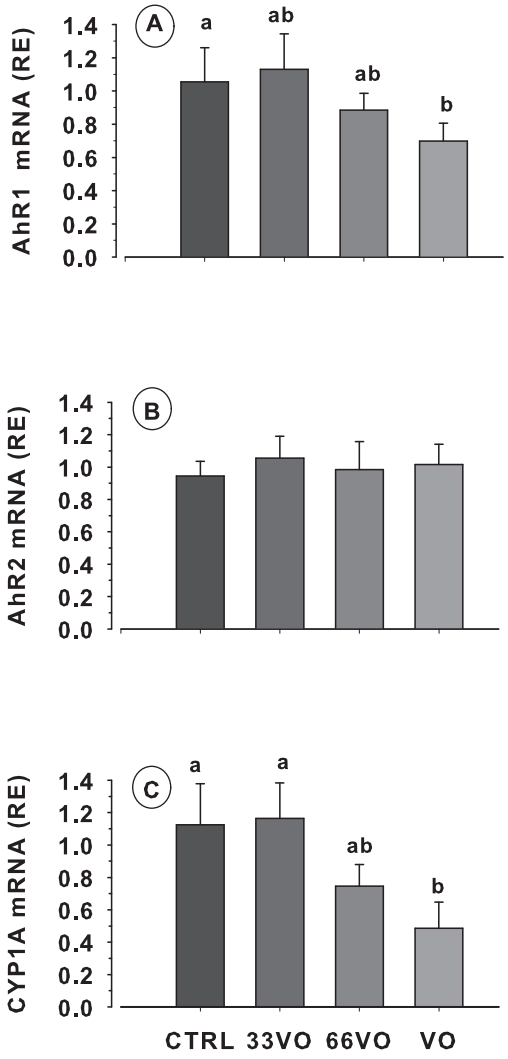

FIG. 1

Figure 2

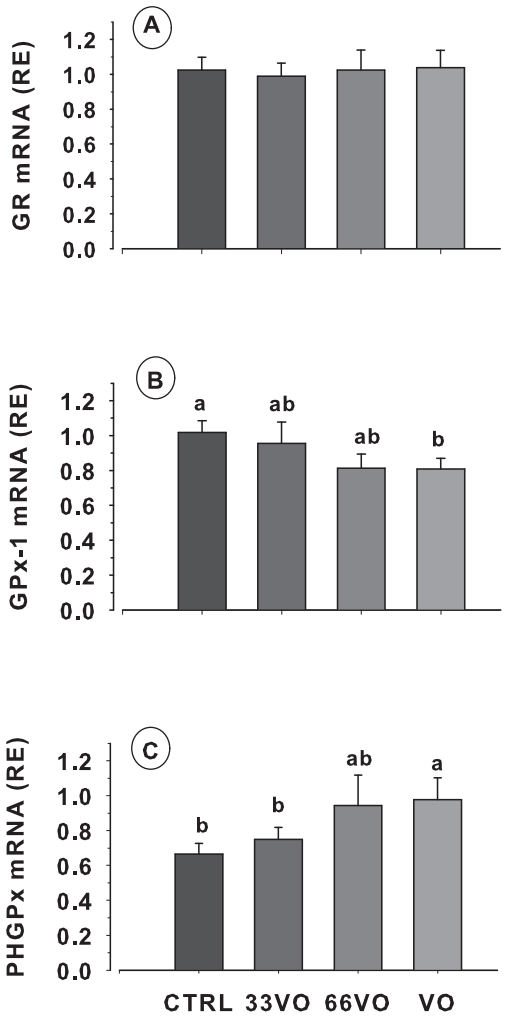

FIG. 2

Figure 3

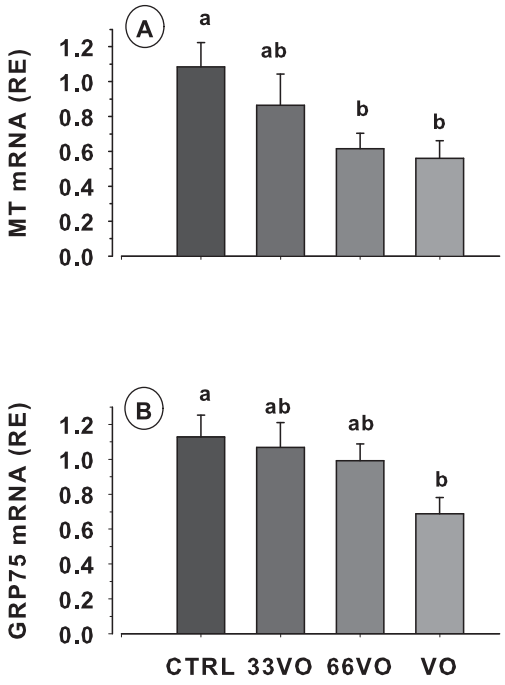

FIG. 3

Figure 4

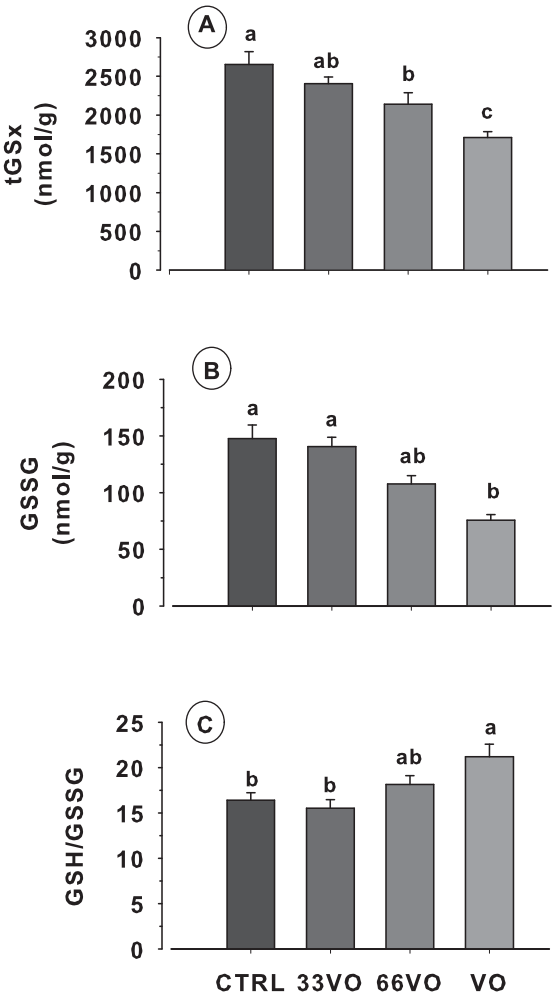

FIG. 4

Figure 5

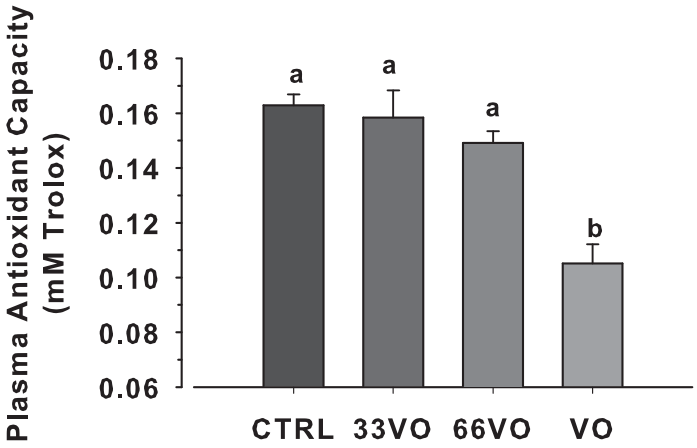

FIG. 5

Figure 6

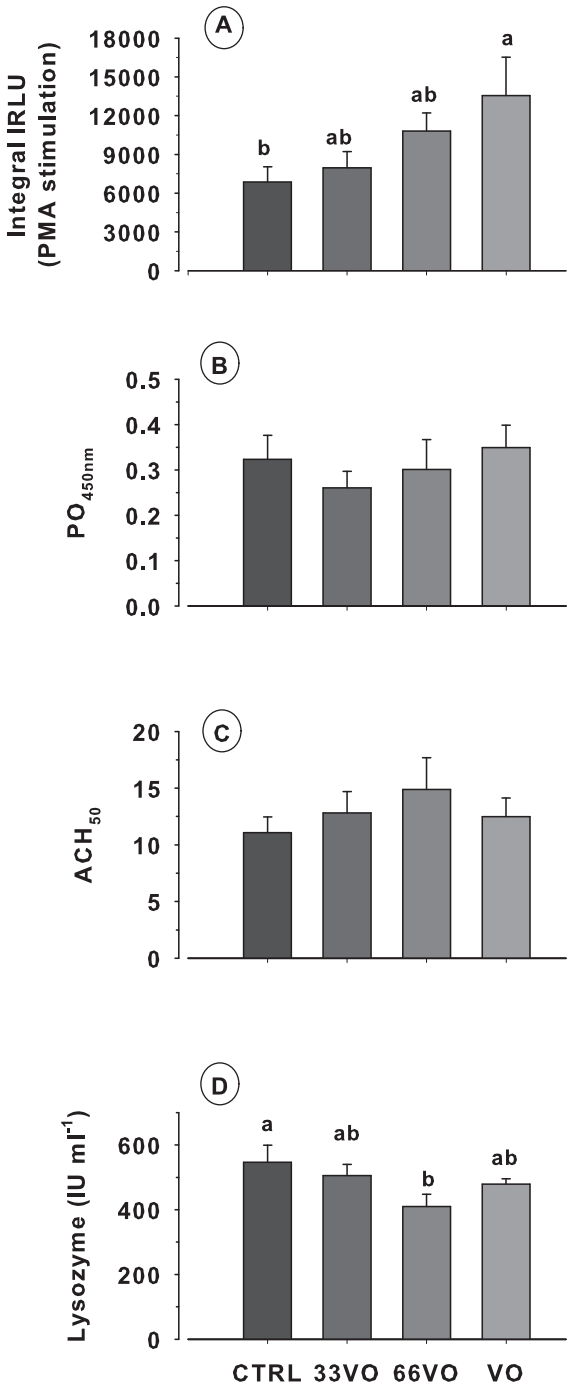

FIG. 6